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Anne Holleran
AU: 1642
Tel: 308-8892
RM: 8e03

mailbox: 8e12

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Enhancement of Antibody Dependent Cellular Cytotoxicity (ADCC) by Combination of Cytokines

DIMITRI FLIEGER, ULRICH SPENGLER, IMKE BEIER, ROLF KLEINSCHMIDT, ANJA HOFF, MICHAEL VARVENNE, TILMAN SAUERBRUCH, and INGO SCHMIDT-WOLF

ABSTRACT

Monoclonal antibodies (MAb) specific for tumor-associated antigens (TAA) can induce an immunological cellular attack of tumor cells by a process termed antibody dependent cellular cytotoxicity (ADCC). Cytokines may augment ADCC by direct activation of immune cells or by enhancement of TAA on tumor cells. Thus, we investigated whether ADCC by MAb 17-1A and BR55-2, which recognize TAA on colorectal tumor cells, can be augmented by 3-day incubation with different concentrations of IL-2, IL-4, IL-6, IL-12, IFN-α, IFN-γ, GM-CSF, M-CSF, and TNF-α. ADCC was assessed by a new flowcytometric cytotoxicity assay (Flieger et al. Immunol Methods 1995; 180:1–13) using PKH-2 labeled HT29 cells as targets and PKH-26 labeled peripheral blood mononuclear cells from three healthy volunteers as effector cells. We found three reaction patterns with the cytokines tested: (a) cytokines, which increase ADCC (IL-2, IL-12, IFN-α, and IFN-γ, which represent Th1 cytokines); (b) cytokines with no effect (GM-CSF, M-CSF, and TNF-α); and (c) cytokines, which decrease ADCC (IL-4 and IL-6, which represent Th2 cytokines). Then, we tested cytokines that increase ADCC in combination with the other cytokines. We found that the combinations IL-2/IFN-α, IL-2/IFN-γ, IL-2/IL-12, and IL-12/IFN-α potentiated ADCC. By contrast, IL-4 reduced the IL-2, IL-12, and IFN-α-induced ADCC. Since the Th1 response, cooperation of monocytes and CD4 cells is involved, we plan to elucidate by magnetic cell sorting (MACS) separation techniques, which cells are involved in cytokine-induced ADCC. Our results may be useful for finding combinations of cytokines and MAb for the locoregional treatment of colorectal cancer.

INTRODUCTION

The availability of monoclonal antibodies (MAb) has broadened the immunological approach for treatment of human cancer. In the treatment of minimal residual disease, encouraging results were obtained with MAb 17-1A in patients with resected Dukes' C colorectal carcinoma reducing the overall death rate by 30% after a mean follow-up of 5 years.[1] In the treatment of advanced disease, first encouraging results were reported for patients with low-grade non-Hodgkin's lymphoma treated with IDEC-C2B8, an anti-CD20 MAb with an overall response of 40%.[2] However, a potentiation of the effects of MAb by combination with other immunomodulatory substances is highly desirable. Cytokines can increase immune cell effector functions[3–5] and can upregulate the expression of tumor-associated antigens (TAA).[6] Therefore, we investigated whether nine different cytokines can influence antibody dependent cellular cytotoxicity (ADCC) of MAb 17-1A and BR55-2, which recognize TAA on colorectal tumor cells. As monocytes/macrophages are an important population exerting ADCC, we measured ADCC by a new flow cytometric method, which also takes into account the effect of long-term ADCC of macrophages.[7]

MATERIALS AND METHODS

Medium and cells

RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 μg/mL streptomycin, 200 IU/mL penicillin and 300 μg/mL L-glutamine was used throughout. The colon carcinoma cell line HT29 (ATCC) was kept in exponential growth conditions in 12.5 mL medium in plastic 25-cm^2

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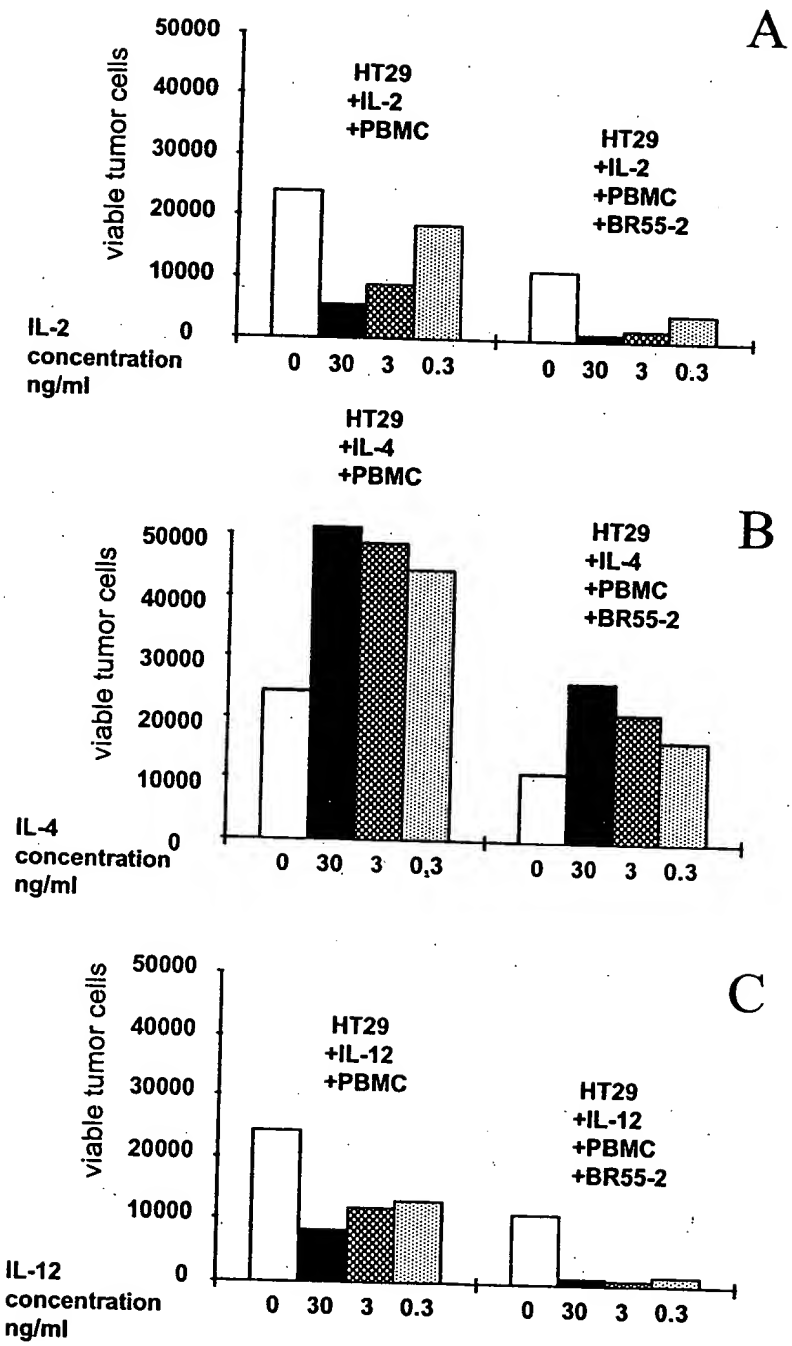

FIG. 1. Effect of various concentrations of cytokines on ADCC mediated by MAb BR55-2. Tumor cells were incubated with peripheral blood mononuclear cells at an effector to target ratio of 270,000 to 30,000 (9:1). Bars represent the means of viable tumor cells obtained in triplicate experiments. The left block of each diagram displays the number of viable cells without or with the addition of various concentrations of cytokines as indicated. The right block of each diagram displays similar conditions as in the left block plus mAb BR55-2 (50 μg/mL). The results of one of three tested healthy effector cell donors are presented.

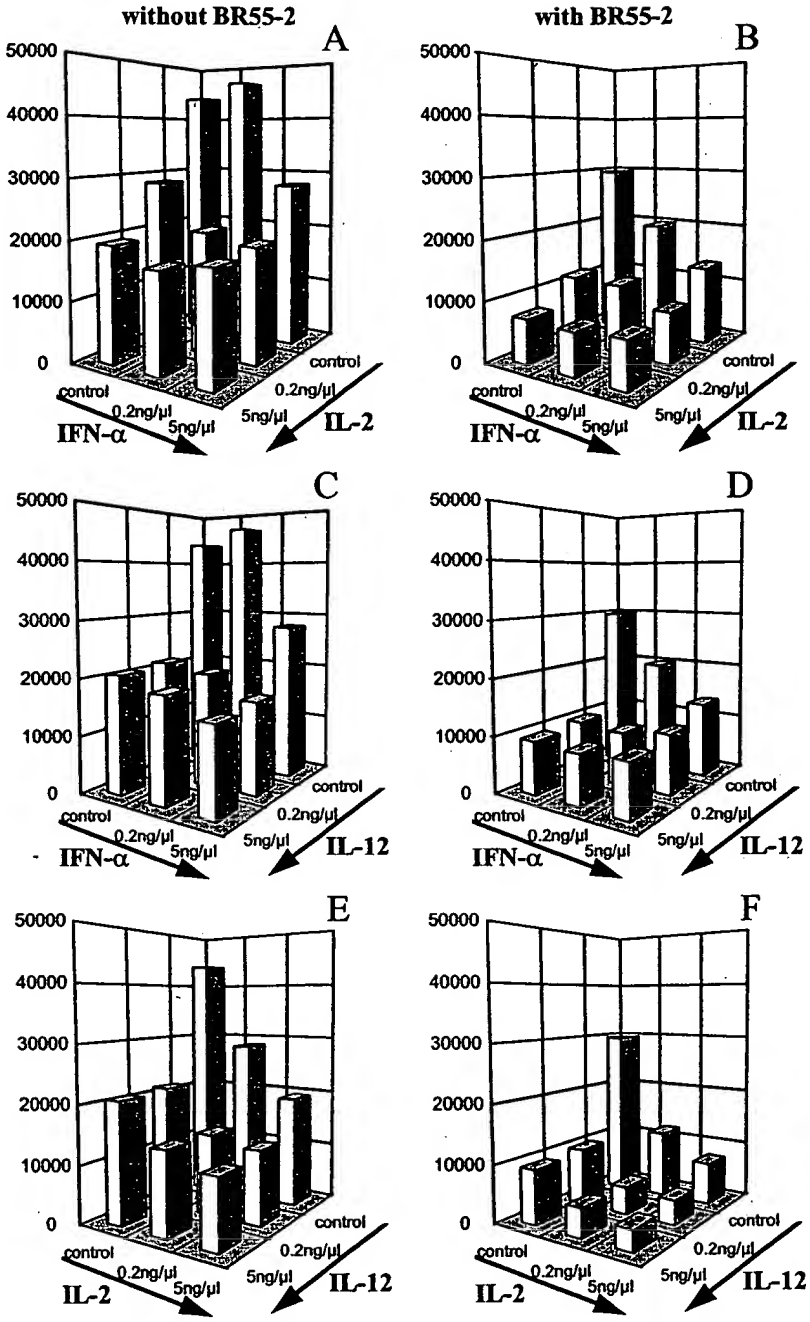

FIG. 2. Enhancement of ADCC of MAb BR55-2 by combination of cytokines. Experimental conditions were identical to those for Figure 1. Bars represent the means of viable tumor cells obtained in triplicate experiments. Each diagram represents combination of cytokines i.e. IFN-α/IL-2 (A,B), IFN-α/IL-12 (C,D) and IL-2/IL-12 (E,F) at two concentrations (i.e., 0.2 and 5 ng/mL). Diagrams to the left (A,C,E) present cultures without MAb and diagrams to the right (B,D,F) cultures treated with 50 μg/mL MAb BR55-2 (results of one representative donor out of three).

culture flasks (Greiner, Frickenhausen, Germany). Detachment of the cells was performed using a rubber policeman.

Cell separation and storage

PBMC were prepared from healthy donors' buffy coat diluted in phosphate buffered saline (PBS) by Ficoll–Hypaque density gradient centrifugation, according to routine procedures.[8] After controlled freezing in medium containing 10% heat-inactivated FCS and 10% dimethylsulfoxid, aliquots were stored in liquid nitrogen.

Monoclonal antibodies

MAb 17-1A of the IgG2a isotype[9] was obtained from Centocor (Malvern, PA). Murine BR55-2 of the original isotype[10] IgG3 was kindly provided by H. Loibner (Novartis, Basel, Switzerland).

Cytokines

IFN-γ (10×10^6 U/mg), IL-2 (5×10^6 U/mg), IL-6 (1×10^7 U/mg), IL-10 (100 ng/mL), M-CSF (55×10^6 U/mg), and GM-CSF (10×10^6 U/mg) were all purchased from IC Chemikalien (Ismaning, Germany). IFN-α (200×10^6 U/mg) and IL-4 (1×10^8 U/mg) were obtained from Pharma Biotechnologie (Hannover, Germany). TNF-α (50×10^6 U/mg) was kindly provided by Bender (Vienna, Austria). IL-12 (2.4×10^8 U/mg) was kindly provided by Hoffmann La-Roche (Grenzach-Wyhlen, Germany).

Cytotoxicity assay

The cytotoxicity assay was performed as previously described.[7] Briefly, PBMC were thawed, washed three times in medium and once in PBS, and then incubated with the red membrane dye PKH-26 (Sigma Chemicals, St. Louis, MO) at 2×10^6 mol/L for 4 min; during this time, the reaction tubes were agitated slightly at room temperature. HT29 tumor cells were harvested with a rubber policeman, washed in PBS and incubated with PKH-2 (green fluorescence) at 2×10^6 mol/L for 10 min. Staining was stopped by addition of FCS and the labeled cells were washed separately three times with medium. Viability (>95%) and cell counts were determined with trypan blue in a Neubauer chamber and uniform cell labeling was ascertained by UV fluorescence microscopy. HT29 tumor cells and PBMC were seeded in 96-well flat bottom microtiter plates (Nunc, Denmark) at an effector to target (E:T) ratio of 9:1 (270,000 PBMC and 30,000 HT29 cells per well). For ADCC cultures, MAbs were added at an optimal final concentration of 50 μg/mL, as determined by preliminary titration experiments; the final volume was always 200 μg/mL and triplicates were used throughout. After an incubation period of 3 days at 37°C and 5% CO_2 in a humidified atmosphere, the plates were washed with PBS, treated with 50 μL warm ethylenediaminetetraacetic acid (EDTA) 0.02% trypsin 0.05% in PBS per well for 10 min and agitated on a plate shaker for 1 min. Thereafter, 200 μL PBS containing 45% FCS for trypsin blocking, propidium iodide (12.5 μg/mL) for labeling of dead cells, and fluorescein isothiocyanate (FITC)-labeled lymphocytes (150,000 cells/mL, i.e., 30,000 cells/200 μL) as standards for determination of cell counts, were added to the detached cells. The lymphocytes were obtained from a patient suffering from chronic lymphocytic leukemia (CLL). All samples were analyzed by flow cytometry using identical gates and instrument settings. Gates were set for the CLL standards and for viable tumor cells, as described[7]. The number of viable tumor cells in each well was calculated using the formula:

$$\text{viable tumor cells} = \frac{\text{events (tumor cell gate)}}{\text{events (standard cell gate)}} \times 30{,}000$$

(30,000 = Number of standard cells added to each well)

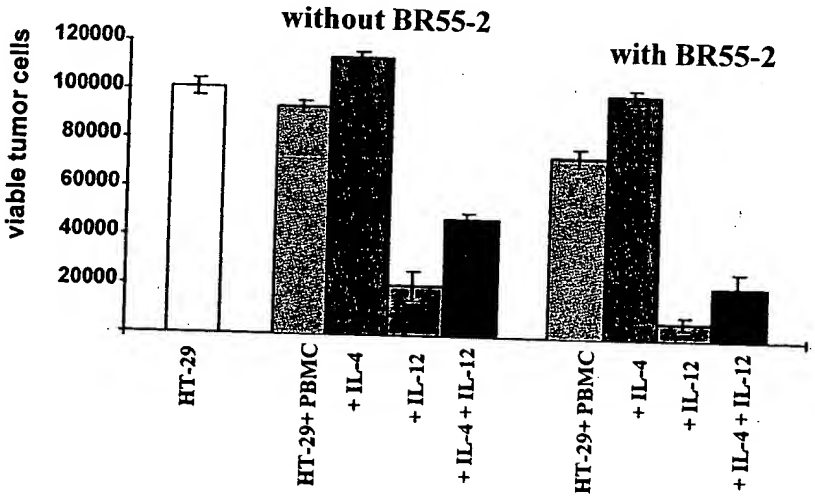

FIG. 3. Suppression of IL-12 induced-ADCC of MAb BR55-2 by the cytokine IL-4. Experimental conditions were identical to those for Figure 1. Bars represent the means of viable tumor cells obtained in triplicate experiments with standard error. The left block of the diagram presents the effect of IL-4 (5 ng/mL) alone, IL-12 (5 ng/mL) alone and the combination IL-4/IL-12 on the PBMC mediated unspecific cytotoxicity. The right block of the diagram displays similar conditions as in the left block plus 50 μg/mL MAb BR55-2 (results of one representative donor out of three).

RESULTS

First we investigated whether single cytokines can enhance ADCC of MAb 17-1A and BR55-2 against the colorectal tumor cell line HT29. The experiments were performed with the PBMC of three normal donors at an effector to target ratio of 9:1, which was found optimal in previous experiments. All cytokines were tested in the concentrations 0.3, 3, and 30 ng/mL. For the cytokines IFN-α, IFN-γ, IL-2, GM-CSF, M-CSF, and TFN-α, we already had shown that IFN-α, IFN-γ, IL-2 significantly enhance the ADCC by MAb 17-1A and BR55-2, whereas GM-CSF, M-CSF, and TNF-α had no effect.[11] Similar experiments were performed for IL-4, IL-6, and IL-12. The cytokine IL-12 significantly enhanced ADCC by the MAb BR55-2 (Fig. 1C). On the contrary, IL-4 reduced ADCC (Fig. 1B). Similar results were obtained with the MAb17-1A (data not shown). The cytokine IL-6 had divergent effects on ADCC with partial inhibition of ADCC or no effect. The direct effect of cytokines on the proliferation of the tumor cell line HT29 was minimal (data not shown).

In further experiments we evaluated whether combination of the three effective cytokines IFN-α, IL-2, and IL-12 with the other cytokines can result to increased ADCC. Here suboptimal concentrations of cytokines were used (i.e., 0.2 and 5 ng/mL) to register additive effects of the cytokines. The combinations IL-2/IFN-α, IL-2/IFN-γ, IL-2/IL-12, and IL-12/IFN-α potentiated significantly ADCC of MAb BR55-2 (one representative normal donor is depicted in Fig. 2) and of MAb 17-1A. The inhibitory cytokine IL-4 suppressed in the contrary significantly the augmentation of ADCC by the cytokines IFN-α, IL-2, and IL-12 (Fig. 3: one representative example for IL-12/IL-4 and BR55-2). Similar results were obtained with both MAbs.

DISCUSSION

The enhancement of ADCC of MAbs specific for TAA by cytokines has already been investigated for the cytokines IFN-α,[3,5] IFN-γ,[5,12] IL-2,[4,13] M-CSF,[14–16] and GM-CSF.[17,18] However, the results were partially inconsistent. Moreover, only one part of the known cytokines were examined and short-term cytotoxicity assays (i.e., chromium release assay and MTT assay) were used.

Therefore, we examined the effect of cytokines in ADCC with a new flowcytometric cytotoxicity test,[7] which also takes into account the long-term cytotoxicity of monocytes/macrophages (72 h). In the single cytokine experiments we found that the ADCC of MAb 17-1A and BR55-2 can be significantly augmented by the cytokines IFN-α, IFN-γ, IL-2, and IL-12.

Data concerning the combination of cytokines are scarce. Increased ADCC was obtained by the combination IFN-α/IL-2[19,20] and IL-2/TNF-α.[21,22] In our experiments we found that the cytokine combinations IL-2/IFN-α, IL-2/IFN-γ, IL-2/IL-12, and IL-12/IFN-α lead to increased ADCC, which is higher than with the single cytokines. In contrast, the cytokine IL-4 suppressed the cytokine-induced ADCC of IFN-α, IL-2, and IL-12. This is the first report describing this effect. Interestingly, IFN-α, IL-2, and IL-12 fit to Th1 cytokines and IL-4 to a Th2 cytokine.[23,24] Thus, since CD4 lymphocytes may be involved in the enhancement or suppression of ADCC, we are now performing magnetic cell sorting (MACS) separation of PBMC subsets and mixing experiments with these subsets to elucidate their effect on ADCC.

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Address reprint requests to:
Dimitri Flieger
Medizinische Universitätsklinik
Allgemeine Innere Medizin
Sigmund-Freud-Str. 25
D-53105 Bonn
Germany

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